Recruitment of Protein Phosphatase 1 to the Nuclear Envelope by A-Kinase Anchoring Protein AKAP149 Is a Prerequisite for Nuclear Lamina Assembly

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Abstract. Subcellular targeting of cAMP-dependent protein kinase (protein kinase A [PKA]) and of type 1 protein phosphatase (PP1) is believed to enhance the specificity of these enzymes. We report that in addition to anchoring PKA, A-kinase anchoring protein AKAP149 recruits PP1 at the nuclear envelope (NE) upon somatic nuclear reformation in vitro, and that PP1 targeting to the NE is a prerequisite for assembly of B-type lamins. AKAP149 is an integral membrane protein of the endoplasmic reticulum/NE network. The PP1-binding domain of AKAP149 was identified as K^{153}GVLF^{157}. PP1 binds immobilized AKAP149 in vitro and coprecipitates with AKAP149 from purified NE extracts. Affinity isolation of PP1 from solubilized NEs copurifies AKAP149. Upon reassembly of somatic nuclei in interphase extract, PP1 is targeted to the NE. Targeting is inhibited by a peptide containing the PP1-binding domain of AKAP149, abolished in nuclei assembled with membranes immunodepleted of AKAP149, and restored after reincorporation of AKAP149 into nuclear membranes. B-type lamins do not assemble into a lamina when NE targeting of PP1 is abolished, and is rescued upon recruitment of PP1 to the NE. We propose that kinase and phosphatase anchoring at the NE by AKAP149 plays in a role in modulating nuclear reassembly at the end of mitosis.

Key words: AKAP • mitosis • nuclear envelope • protein kinase A • protein phosphatase 1

Introduction

The nuclear envelope (NE)[1] is a highly dynamic structure that reversibly breaks down at mitosis (Collas and Courvalin, 2000). The NE consists of a double membrane fenestrated by nuclear pores and underlaid by the nuclear lamina. The outer nuclear membrane is continuous with the ER and shares biochemical and functional properties with the ER. The inner nuclear membrane harbors specific integral proteins that provide attachment sites for chromatin and the lamina (Wilson, 2000; Worman and Courvalin, 2000). Intermediate filaments called A/C- and B-type lamins make up the nuclear lamina. Lamins are believed to mediate interactions between the inner nuclear membrane and chromatin, participate in DNA replication (Ellis et al., 1997; Spann et al., 1997), and may provide a structural role for RNA splicing (Jagatheesan et al., 1999). Disassembly of the NE at mitosis correlates with phosphorylation of proteins of the nuclear membranes, nuclear pores, lamina, and chromatin. In late anaphase, ER-derived membranes associate with chromatin to reform nuclear membranes (Chaudhary and Courvalin, 1993) and in telophase, lamins are dephosphorylated and repolymerize into a lamina (Ottaviano and Gerace, 1985).

Protein phosphatase type 1 (PP1) has been identified as a mitotic lamin B phosphatase (Thompson et al., 1997). PP1 constitutes a family of Ser/Thr phosphatases highly conserved among eukaryotes. It consists of a catalytic subunit associated with a regulatory and targeting subunit (Zolnierowicz and Bollen, 2000). PP1 regulates several processes in mammalian cells, including maintenance of the tumor suppressor retinoblastoma (Rb) in an active form (Ludlow et al., 1993), spliceosome assembly (Mermoud et al., 1994), histone H1 dephosphorylation (Paulson et al., 1996), and mitosis exit (Fernandez et al., 1992). The multiplicity of PP1 functions implies that the PP1 isoforms achieve specificity through subcellular targeting by their regulatory subunits (Zolnierowicz and Bollen, 2000).

Activities of type II cAMP-dependent protein kinase (protein kinase A [PKA]) and A-kinase anchoring pro-

teins (AKAPs) have been implicated in the regulation of nuclear and chromosome dynamics at mitosis (Lamb et al., 1991; Collas et al., 1999; Steen et al., 2000). AKAPs ensure subcellular targeting of the RII regulatory subunit of PKA and are believed to enhance specificity of PKA activity (for review see Colledge and Scott, 1999). Few AKAPs associated with nuclear compartments have been identified. The developmentally regulated nAKAP150 has been identified in the nuclear matrix of precartilage nuclei (Zhang et al., 1996). Another nuclear matrix–associated AKAP, AKAP95 (Eide et al., 1998), has been shown to bind chromosomes at mitosis and to be implicated in chromosome condensation (Collas et al., 1999). Finally, a 255-kD membrane-bound AKAP has been shown to target PKA to the NE of differentiated myocytes (Kapiloff et al., 1999). Whether the NE harbors other AKAPs and the significance of such associations have not been investigated.

S-AKAP84 (Lin et al., 1995), D-AKAP1 (Huang et al., 1997), AKAP121 (Chen et al., 1997), and AKAP149 (Trendelenburg et al., 1996) represent AKAP isoforms that arose by alternative splicing. These isoforms share a 525–amino acid NH_2-terminal core but differ in their COOH-terminal domain. S-AKAP84, D-AKAP1, and AKAP149 have been shown or suggested to be targeted to mitochondria. However, two alternative NH_2-terminal splice variants of D-AKAP1 direct this AKAP to mitochondria or to the ER (Huang et al., 1999). Moreover, in assessing PKA-binding proteins of the NE, we have identified in a PKA-RII overlay assay an ~150-kD AKAP in purified NE extracts from HeLa cells, which reacted with anti-AKAP149 mAbs (see Fig. 1 A). This suggested that AKAP149 is not restricted to mitochondria but might also be targeted to the ER/NE network.

Using cell fractionation studies, membrane vesicle reconstitutions, a novel in vitro nuclear reassembly assay, and competition experiments, we demonstrate here that AKAP149 is an integral protein of the ER/NE membrane network. At the NE, AKAP149 recruits PP1 upon nuclear reassembly in vitro. PP1 anchoring at the NE correlates with assembly of B-type lamins. These findings support a model in which AKAP149 anchors a protein phosphatase implicated in NE reassembly at the end of mitosis.

Materials and Methods

Antibodies

Anti-AKAP149 mAbs raised against amino acids 66–212 of AKAP149 and anti-AKAP95 mAbs were from Transduction Laboratories. Antibodies against calnexin (goat polyclonal G-20) and against PP1 (rabbit polyclonal FL-18, and mAb E-19) were from Santa Cruz Biotechnology, Inc. The anti-PP1 mAb was specific for all PP1 catalytic subunits. Affinity-purified rabbit antibodies against human lamin B receptor (LBR) and against a peptide of human lamin B (gifts from B. Buendia and J.-C. Courvalin, Institut J. Monod, Paris, France) were as described (Chaudhary and Courvalin, 1993; Buendia and Courvalin, 1997). Note that the anti-lamin B antibody did not distinguish between the different human lamin B isoforms (these are referred to as lamin B in the text).

Cells, Nuclei, and Nuclear Fractionation

HeLa cells were grown and synchronized in M phase with nocodazole as described previously (Eide et al., 1998). Interphase nuclei were isolated from unsynchronized HeLa cells by Dounce homogenization and sedimentation as described elsewhere (Collas et al., 1999).

High salt–extracted nuclear matrices were prepared as described elsewhere (Steen et al., 2000). Nuclear matrices were defined as nuclear scaffolds resistant to extraction with 1 mg/ml DNase I, 0.5% Triton X-100, and 2 M NaCl. Isolation of interphase chromatin was carried out as described previously (Collas et al., 1999). Permeabilized nuclei were digested with 5 U micrococcal nuclease, sedimented, the supernatant was collected, and the pellet was incubated in 2 mM EDTA. After sedimentation, the second supernatant was combined with the first to yield a soluble chromatin fraction. Proteins were precipitated with trichloroacetic acid and dissolved in SDS sample buffer.

NEs were purified from interphase HeLa cells essentially according to Dwyer and Blobel (1976) except that only one step of DNase/RNase digestion was performed. NEs prepared under these conditions contained both nuclear membranes and the lamina.

Cell Extracts

Mitotic cytosolic extracts were prepared from synchronized mitotic HeLa cells as described previously (Collas et al., 1999). Cells were washed in lysis buffer, packed, resuspended in 1 vol of lysis buffer, homogenized by sonication, and centrifuged at 10,000 g for 10 min. The supernatant was cleared at 200,000 g for 2.5 h at 4°C in a Beckman SW55Ti rotor. The clear supernatant (mitotic cytosol) was collected, aliquoted, and frozen. Interphase extracts were prepared as above from unsynchronized HeLa cells (95–98% in interphase) except that EDTA was omitted from the lysis buffer.

Membrane Vesicle Isolation and Fractionation

Mitotic membrane vesicles were recovered from the 200,000-g pellet after mitotic cytosol preparation, washed by resuspension and sedimentation at 100,000 g for 30 min in membrane wash buffer (MWB: 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 50 mM Hepes, pH 7.5, 1 mM DTT, 1 mM ATP, and protease inhibitors), frozen in liquid nitrogen, and stored at −80°C. Protein concentration of the vesicle fractions was 10 mg/ml. Vesicles prepared from interphase cells were used in fractionation studies. Vesicles were isolated from 10,000-g interphase cell extracts by sedimentation at 200,000 g onto a 2 M sucrose cushion. Vesicles were collected, washed, and resuspended in membrane wash buffer containing 2 M sucrose. Vesicles were fractionated by floatation to density equilibrium into a 2.0–0.2 M sucrose gradient at 150,000 g in a Beckman SW28 rotor for 24 h at 4°C. 20 200-μl fractions were recovered and solubilized in SDS sample buffer.

Nuclear Assembly Assay

A condensed chromatin substrate for nuclear reassembly was prepared by disassembling purified HeLa nuclei in mitotic cytosol as described previously (Collas et al., 1999). Condensed, membrane-free chromatin masses were recovered by sedimentation through a 1 M sucrose cushion and resuspended in interphase cytosol at 4–5,000 chromatin U/μl. The cytosol was supplemented with mitotic membranes to provide vesicles harboring integral membrane proteins required for NE assembly (see Results) (Pyrpasopoulou et al., 1996). An ATP-generating system (2 mM ATP, 20 mM creatine phosphate, 50 μg/ml creatine kinase) and 100 μM GTP were added to promote chromatin decondensation, nuclear membrane vesicles binding to chromatin, and fusion (Collas et al., 1996). The reaction was incubated at 30°C for up to 2 h, and nuclear reassembly was monitored by phase–contrast microscopy, DNA staining with 0.1 μg/ml Hoechst 33342, and immunofluorescence analysis of NE markers. In some experiments, the interphase cytosol was preincubated with competitor peptides for 15 min at 4°C before adding the ATP-generating system.

Immunodepletion of AKAP149 and Reconstitution of Membrane Vesicles

Mitotic membranes (10 mg/ml protein) were solubilized with 0.5% NP-40 in MWB adjusted to 400 mM KCl (MWB/KCl) for 30 min at 4°C. The extract was centrifuged at 15,000 g for 15 min to sediment the nonsolubilized material. AKAP149 was immunodepleted from the clarified detergent extract using anti-AKAP149 mAbs coupled to protein A–Sepharose beads as described below. Control immunodepletions were done using nonimmune mouse IgGs. AKAP149-depleted and mock-depleted vesicles were reconstituted by dialyzing the detergent out of the unbound material against KHM (50 mM KCl, 50 mM Hepes, pH 7.5, 4 mM $MgCl_2$, 10 mM

EGTA, 10 mM CaCl$_2$, 1 mM DTT) overnight at 4°C (Pyrpasopoulou et al., 1996). After dialysis, vesicles were diluted with 1 vol of MWB, sedimented at 150,000 g, resuspended in MWB, aliquoted, and frozen. Immunodepletion of AKAP149 was verified by Western blotting analysis.

Immunological Procedures

Western blotting analysis was performed as described previously (Collas et al., 1999) using anti-AKAP149 antibodies (1:250 dilution), anticalnexin (1:500), anti-PP1 (1:500), and peroxidase-conjugated secondary antibodies. For immunoprecipitations, whole mitotic or interphase cells were sonicated in immunoprecipitation buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1% Triton X-100, and protease inhibitors) and the lysate was centrifuged at 15,000 g. After preclearing the supernatant, immunoprecipitations were carried out with relevant antibodies (diluted 1:50) at room temperature for 2.5 h, followed by incubation with protein A–Sepharose for 1 h and centrifugation at 4,000 g. Immune complexes were washed, and proteins were eluted in SDS sample buffer (Collas et al., 1999). AKAP149 was immunodepleted from NP-40–solubilized mitotic vesicles as described above. Immunofluorescence analysis of cells, nuclei, and chromatin was performed as described earlier (Collas et al., 1999). Specimens were fixed with 3% paraformaldehyde and permeabilized with 0.5% (cells) or 0.1% (nuclei) Triton X-100. Alternatively and as indicated, samples were fixed with −20°C methanol for 5 min. Primary and secondary antibodies (FITC or TRITC conjugated) were used at a 1:100 dilution. DNA was stained with 0.1 μg/ml Hoechst 33342. Observations were made on an Olympus BX60 microscope, and photographs were taken with a JVC camera and AnalySIS software (Soft Imaging Systems).

RII and PP1 Overlays

AKAP149 immune precipitates were resolved by SDS-PAGE and immobilized onto nitrocellulose. RII binding was detected using recombinant ^{32}P-labeled human RIIα in an overlay assay (Bregman et al., 1989). PP1 binding to immobilized AKAP149 immune precipitates was detected on a PP1 overlay. The membrane was incubated with 0.7 U recombinant PP1 (Upstate Biotechnology) for 2 h at room temperature in TBST, washed three times for 5 min in TBST, and PP1 binding was detected by Western blotting using anti-PP1 mAbs.

Microcystin-Sepharose Affinity Purification of PP1

HeLa cells were harvested and lysed in immunoprecipitation buffer. Cell lysates (250 μl; 4 mg total protein) were precleared with protein A–Sepharose beads for 30 min at 4°C and incubated with 25 μl of a 50% slurry of microcystin-Sepharose beads (Upstate Biotechnology) for 1 h at room temperature. Beads were washed with buffer M (50 mM triethanolamine, pH 7.5, 0.1 mM EGTA, 5% glycerol, 0.5 M NaCl, 0.1% β-mercaptoethanol, and protease inhibitors) to remove unbound proteins. Affinity-purified PP1 and bound proteins were eluted with 3 M NaSCN in buffer M. Input lysate, unbound fractions, and bound eluates were subjected to immunoblotting analysis. Control samples were incubated with protein A–Sepharose beads and processed as above. PP1 was also affinity-purified from purified NEs (5–10 × 10^7) solubilized with 1% Triton X-100/1 M NaCl.
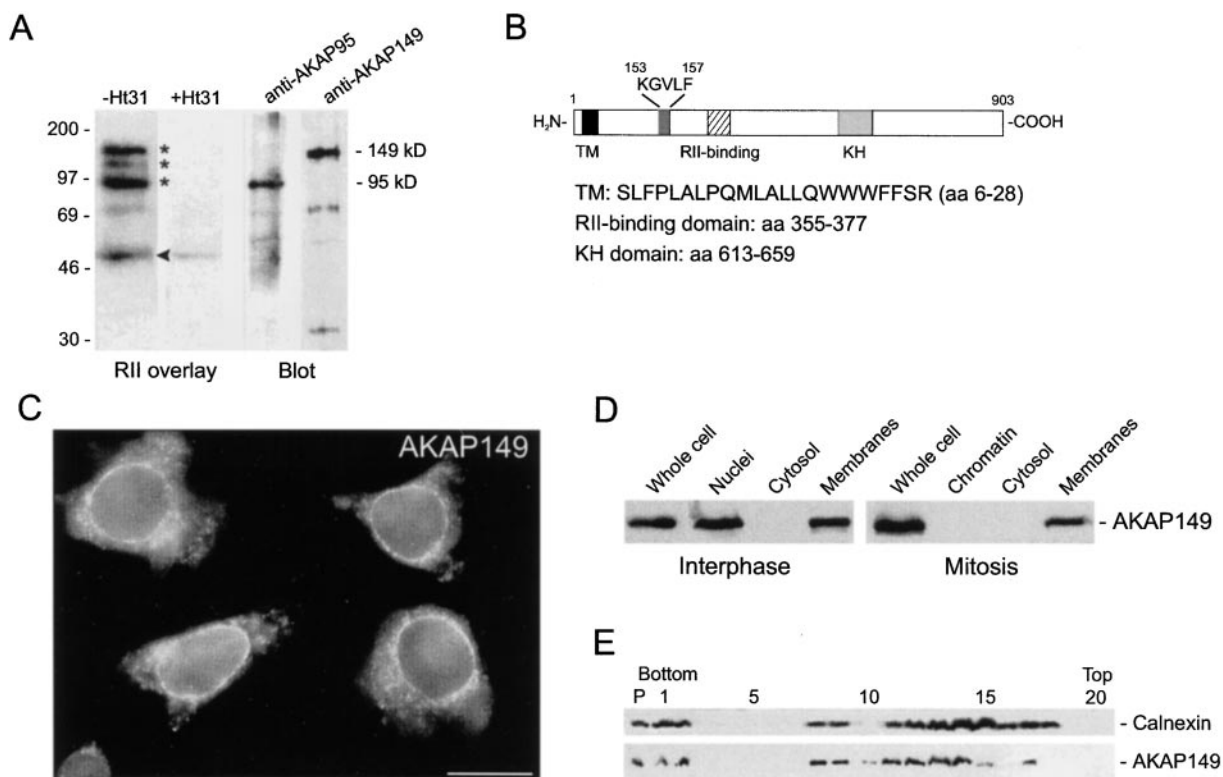
Figure 1. AKAP149 is a protein of the ER/NE network. (A) RII overlay of HeLa cell NE proteins using ^{32}P-labeled RIIα as a probe without or with 100 nM Ht31 competitor peptide (left). Asterisks indicate RII-binding proteins; arrowhead points to RII. The filters were also immunoblotted using anti-AKAP95 and anti-AKAP149 mAbs (right). (B) Structural domains of AKAP149. TM, predicted transmembrane domain. KGVLF represents a putative PP1-binding domain. AKAP49 also contains PKA-RII–binding and putative RNA-binding (KH) domains. aa, amino acids. (C) Distribution of AKAP149 in unsynchronized HeLa cells was examined by immunofluorescence using the anti-AKAP149 mAb. (D) Interphase and mitotic HeLa cells were fractionated into nuclei or chromatin, cytosol, and cytoplasmic membranes and each fraction was immunoblotted using anti-AKAP149 mAbs. (E) Interphase membrane vesicles were fractionated by floatation in a sucrose density gradient, and fractions were immunoblotted using anticalnexin and anti-AKAP149 antibodies. P, residual pellet. Bar, 10 μm.

Results

AKAP149 Is an Integral Membrane Protein of the ER and of the NE

In a search for AKAPs of the NE, we performed an overlay of immobilized purified HeLa NE proteins with ^{32}P-labeled recombinant human RIIα. In addition to PKA-RIIα (Fig. 1 A, arrowhead), three PKA-binding proteins of apparent $M_r \sim 150$, ~ 130, and 95 kD were identified (Fig. 1 A, asterisks). RIIα binding to each protein was competed with 100 nM of the AKAP-RII anchoring inhibitor peptide Ht31 (Carr et al., 1991), illustrating the specificity of binding. Probing the membrane with an anti-AKAP95 mAb argued that the 95-kD RII-binding protein was AKAP95 (Fig. 1 A, blot), an AKAP of the nuclear matrix and thus expected in our NE preparations. Moreover, the ∼150-kD protein comigrated with AKAP149 when the membrane was probed with an anti-AKAP149 mAb (Fig. 1 A), suggesting that this RII-binding protein was AKAP149. Note that an 80-kD RII-binding polypeptide faintly detected in the overlay comigrated with a peptide detected by anti-AKAP149 mAbs, suggesting that this peptide was a proteolytic product of AKAP149 (Fig. 1 A). The ∼130-kD polypeptide remains unaccounted for to date.

Sequence analysis of AKAP149 revealed that the protein contains one NH$_2$-terminal transmembrane domain (Fig. 1 B; TM at residues 6–28). This is highly consistent with the functional transmembrane domain identified in the AKAP149 splice variants (Lin et al., 1995); Chen et al., 1997; Huang et al., 1997). A PKA type II (RII) anchoring domain and a putative RNA-binding (KH) domain have also been identified (Trendelenburg et al., 1996). AKAP149 also harbors a putative consensus binding site for PP1 (K^{153}GVLF^{157}) (Zhao and Lee, 1997) (Fig. 1 B). These observations suggest that AKAP149 is a transmembrane protein with multiple functional domains.

We examined the subcellular localization of AKAP149 in HeLa cells using the anti-AKAP149 mAb (note that AKAP149 was also detected on immunoblots of human B cell, T cell, epithelial and endothelial cell lines; data not shown). Immunofluorescence analysis of methanol-fixed HeLa cells showed that AKAP149 was primarily cytoplasmic and that the mAbs also decorated the nuclear periphery (Fig. 1 C). Similar results were obtained with paraformaldehyde-fixed cells (data not shown). Furthermore, AKAP149 cofractionated with nuclei and cytoplasmic membranes of interphase cells, and with membranes of mitotic cells (Fig. 1 D). Additionally, when interphase microsomes were fractionated by floatation to density equilibrium in a sucrose gradient, AKAP149 was found to cofractionate with calnexin, a marker of the ER membrane (Fig. 1 E). These results indicate that AKAP149 is a protein of the ER membrane network, and as the outer nuclear membrane is continuous with the ER, detection of AKAP149 in nuclei argues that the AKAP also resides in the NE.

The subnuclear localization of AKAP149 was identified in purified HeLa nuclei fractionated into chromatin, "matrix" (defined as a detergent-, high salt–, and DNase-resistant nuclear extract), and NE (nuclear membrane/lamina fraction). AKAP149 was detected exclusively in the NE by
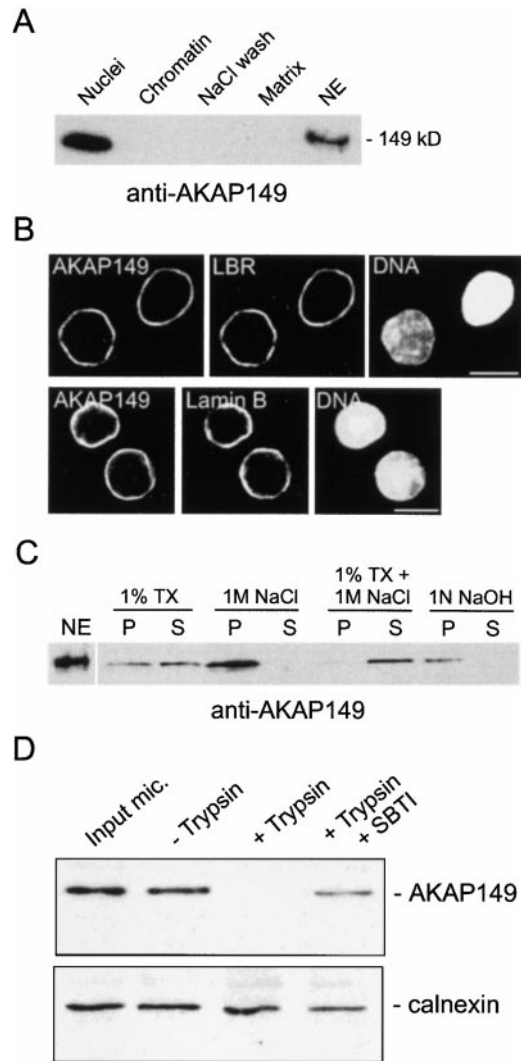
Figure 2. AKAP149 is an integral membrane protein of the NE. (A) Purified HeLa nuclei were fractionated into chromatin, matrices, and NEs and distribution of AKAP149 in each fraction was determined by immunoblotting. (B) Immunofluorescence colocalization of AKAP149 with either LBR (top) or lamin B (bottom) in isolated HeLa nuclei. DNA was labeled with Hoechst 33342. (C) Purified HeLa NEs were extracted with 1% Triton X-100, 1 M NaCl, 1% Triton X-100/1 M NaCl, or 1 N NaOH. Unextracted (P) and extracted proteins (S) were immunoblotted using anti-AKAP149 mAb. (D) Purified HeLa cell microsomes (Input mic.) were exposed to either no trypsin, 100 μg/ml trypsin, or 100 μg/ml trypsin plus 100 μg/ml soybean trypsin inhibitor (SBTI), for 15 min and solubilized in SDS sample buffer. Accessibility of AKAP149 and of the ER protein calnexin to trypsin was analyzed by immunoblotting. Bars, 10 μm.

immunoblotting (Fig. 2 A). NE localization of AKAP149 was confirmed by immunofluorescence analysis of purified nuclei, as shown by colocalization with LBR and lamin B, markers of the inner nuclear membrane and of the nuclear lamina, respectively (Worman et al., 1990) (Fig. 2 B). Additionally, AKAP149 was not extractable from purified NEs with 1 M NaCl or alkaline treatment, but was partially extracted with 1% Triton X-100 and fully extracted with 1% Triton X-100/1 M NaCl (Fig. 2 C). Thus,

AKAP149 exhibits extraction properties of integral membrane proteins, supporting the topology of AKAP149 predicted by its primary structure. We concluded that in addition to being distributed in the ER, AKAP149 is an integral protein of the NE.

The topology of AKAP149 in the ER/NE membrane was determined by assessing the sensitivity of the AKAP149 epitope (localized between amino acids 66 and 212) to trypsin digestion. Immunoblotting analysis shows that treatment of intact microsomes with 100 μg/ml trypsin fully degraded AKAP149, whereas calnexin (whose epitope faces the ER lumen) was not affected (Fig. 2 D). This result indicates that the bulk of AKAP149 faces the cytoplasm.
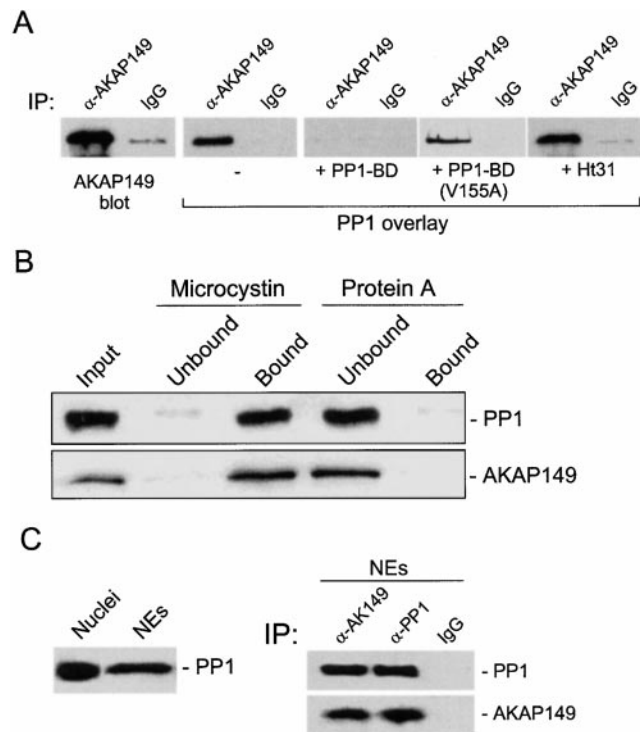
AKAP149 Binds PP1 In Vitro and In Vivo

We have identified a putative binding site for PP1 in the NH$_2$-terminal domain of AKAP49 (see Fig. 1 A). To test whether AKAP149 interacts with PP1, we first determined whether AKAP149 bound PP1 in an overlay assay. An AKAP149 immune precipitate from HeLa cell lysates was immobilized onto nitrocellulose, incubated with recombinant PP1, and PP1 binding was detected by immunoblotting. Fig. 3 A shows that PP1 bound to a 149-kD protein of the AKAP149 immune complex, but not to a control precipitate. PP1 binding was abolished by 10 μM of a competitor peptide containing the putative PP1-binding site of AKAP149 (PP1-BD: SSPKGVLFSS). However, binding was not inhibited by 10 μM of a control PP1-BD(V155A) peptide harboring a Val^{155}→Ala mutation, or by 10 μM of the RII-anchoring inhibitor peptide Ht31 (Fig. 3 A). As the Val→Ala mutation within the PP1-binding domain of PP1 regulatory subunits is known to abolish interaction with PP1 (Beullens et al., 1999), we concluded from these data that AKAP149 bound PP1 in vitro.

Interaction of AKAP149 with PP1 in vivo was examined in affinity purification and immunoprecipitation experiments. Affinity purification of PP1 from interphase cell lysates with the PP1 inhibitor microcystin, coupled to sepharose beads, copurified AKAP149 as shown by immunoblotting of microcystin-Sepharose–bound and unbound fractions (Fig. 3 B). In contrast, neither protein was isolated with control protein A–Sepharose beads (Fig. 3 B). Thus, AKAP149 resides in a complex with PP1 in vivo. To determine whether NE-associated AKAP149 was also associated with PP1, we carried out immunoprecipitations from NE extracts. Immunoblotting analysis of nuclei and purified NEs revealed PP1 in these structures (Fig. 3 C, left). Moreover, AKAP149 and PP1 were coimmunoprecipitated from NE extracts using either anti-AKAP149 or anti-PP1 mAbs (Fig. 3 C, right). These results argue that AKAP149 is associated with PP1 in vivo and may anchor PP1 at the NE.

### Dynamics of Nuclear Membranes, Lamin B, and PP1 during Nuclear Reconstitution In Vitro

To investigate the dynamics of the interaction of AKAP149 with PP1 at the NE, we developed a cell-free assay that recapitulates nuclear reassembly at the end of mitosis. A condensed chromatin substrate was prepared by disassembling purified HeLa nuclei in a mitotic cytosol

Figure 3. AKAP149 binds PP1 in vitro and in vivo. (A) Binding of PP1 to AKAP149. AKAP149 and control immune precipitates (IP) immobilized on nitrocellulose (AKAP149 blot) were incubated with either recombinant PP1 alone, PP1 plus 10 μM competitor peptide PP1-BD, PP1 plus 10 μM control PP1-BD(V155A) peptide, or PP1 plus 10 μM RII-anchoring inhibitor peptide Ht31. PP1 binding was detected by immunoblotting using the anti-PP1 mAb. (B) Affinity purification of PP1 with microcystin-sepharose beads from interphase cell lysates. Immunoblot analysis was carried out on input lysate (Input), microcystin-treated lysate (Unbound), and eluates from microcystin-sepharose beads (Bound) using anti-PP1 and anti-AKAP149 mAbs. Control isolations were performed with protein A–Sepharose beads. (C) Left panel, immunoblot of nuclei and NEs with anti-PP1 mAb. Right panels, AKAP149 and PP1 were immunoprecipitated (IP) from NE extracts and precipitates were immunoblotted using anti-PP1 and anti-AKAP149 mAbs. IgG, control immunoprecipitation with nonimmune mouse IgGs.

(Steen et al., 2000). The condensed chromatin was purified through a sucrose cushion and resuspended in interphase cytosol containing an ATP-regenerating system and exogenous membrane vesicles to promote nuclear assembly.

The distribution of PP1 and AKAP149 was monitored by immunofluorescence during successive stages of nuclear reassembly. As the chromatin decondensed, membrane vesicles were targeted to the chromatin and fused to form nuclear membranes, as shown by phase–contrast and immunofluorescence analysis of the LBR (Fig. 4 A). Nucleoli were also detected in reconstituted nuclei (Fig. 4 A, 120 min), indicating that nuclear import and nucleolus assembly were also recapitulated in the extract. PP1 was associated with input condensed chromosomes (Fig. 4 A, PP1, 0 min) in agreement with earlier observations of mitotic cells (Fernandez et al., 1992). As the chromatin decondensed, PP1 labeling faded (30 min) and sealing of nu-
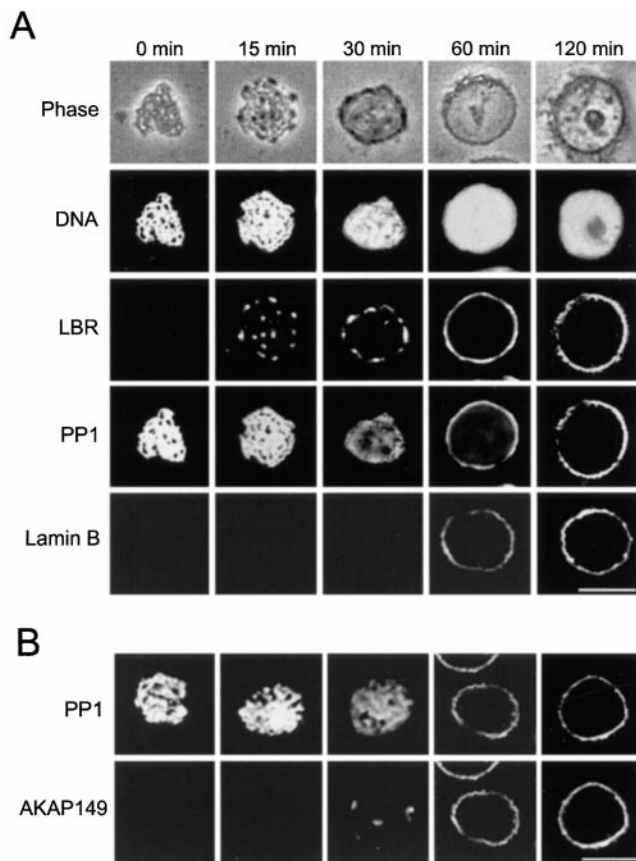
Figure 4. Recruitment of nuclear PP1 to the NE upon nuclear reassembly in vitro. Condensed chromatin substrates were incubated in an interphase extract containing membrane vesicles. (A) Nuclear reassembly was monitored by phase–contrast microscopy (top). DNA was stained with Hoechst 33342. Assembly of nuclear membranes and PP1 distribution were examined by immunofluorescence analysis of methanol-fixed nuclei, using anti-LBR rabbit polyclonal antibodies and anti-PP1 mAbs, respectively. Nuclear lamina assembly was visualized using anti-lamin B antibodies. (B) PP1 distribution and targeting of AKAP149 to the reforming nucleus were monitored by double immunofluorescence using anti-PP1 polyclonal antibodies and the anti-AKAP149 mAb. Note the association of PP1 with the nuclear periphery only when AKAP149 is assembled into the NE (~60 min). Bars, 10 μm.

clear membranes by 60 min coincided with smooth peripheral staining of PP1, suggesting a recruitment of at least some PP1 to the NE. Labeling of PP1 at the NE coincided with assembly of lamin B into the NE (Fig. 4 A). Moreover, colabeling of PP1 and AKAP149 showed that recruitment of PP1 to the NE correlated with assembly of AKAP149 into the NE (Fig. 4 B). Altogether, these observations illustrate the redistribution of a fraction of chromatin-associated PP1 to the NE. They also illustrate a temporal relationship between PP1 recruitment to the NE and AKAP149 assembly into nuclear membranes.

A consistent observation in the above experiment was the fading of intranuclear PP1 labeling during chromatin decondensation and its absence from the nuclear interior in reconstituted nuclei (Fig. 4). Western blotting analysis of equal numbers (10^5) of purified interphase HeLa nuclei,
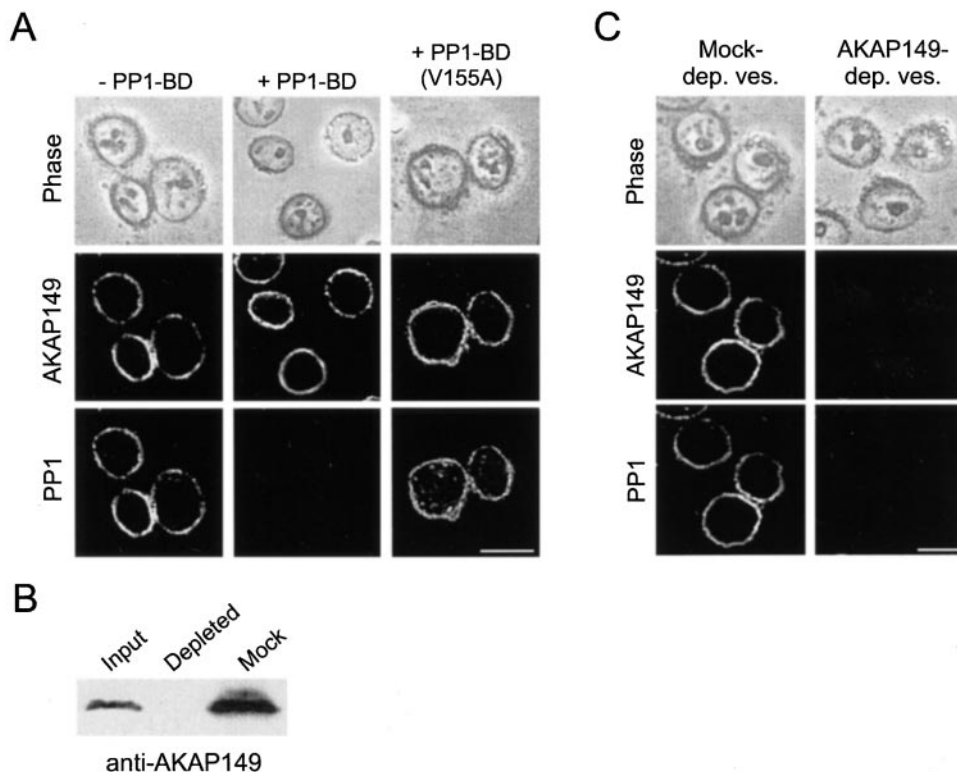
chromatin masses condensed in vitro, and nuclei reconstituted in vitro using the anti-PP1 mAb revealed similar amounts of PP1 in all samples (data not shown). Thus, the disappearance of intranuclear PP1 labeling during nuclear reassembly in vitro resulted from antigen masking rather than from solubilization of the phosphatase.

AKAP149 Recruits PP1 at the NE upon Nuclear Assembly In Vitro

Interaction of PP1 with AKAP149 and localization of PP1 at the nuclear periphery during nuclear reassembly in vitro led to the hypothesis that NE-bound AKAP149 recruits PP1 during nuclear reassembly. To test this, nuclei were reassembled in vitro as above except that the interphase cytosol contained 1 μM of the PP1 binding competitor peptide PP1-BD, or 1 μM of the control PP1-BD(V155A) peptide. Either peptide was preincubated with condensed chromatin on ice for 15 min before induction of nuclear reassembly. After 2 h at 30°C, AKAP149 and PP1 were immunofluorescently examined after methanol fixation. Both control cytosols (containing no peptide or PP1-BD[V155A]) and PP1-BD–containing cytosol promoted assembly of nuclear membranes harboring AKAP149 (Fig. 5 A). However, PP1-BD, but not PP1-BD(V155A), completely abolished PP1 labeling at the nuclear periphery (Fig. 5 A). This was verified with the anti-PP1 mAb (see below, in Fig. 7 A). Therefore, binding of PP1 to AKAP149 is implicated in recruiting of PP1 to the NE.

To provide further evidence for the role of AKAP149 in PP1 recruitment to the NE, we reassembled nuclei using membrane vesicles depleted of AKAP149. AKAP149 was immunodepleted (or mock-depleted) from mitotic membrane vesicles after solubilization with 0.5% NP40/0.4 M KCl, by two rounds of immunoprecipitation using the anti-AKAP149 mAb. Solubilized vesicles were reconstituted after detergent removal by dialysis, vesicles were sedimented and washed, and AKAP149 depletion was assessed on Western blots. AKAP149 was clearly immunodepleted from the vesicles, whereas mock depletions using nonimmune mouse IgGs did not remove the AKAP (Fig. 5 B). Both mock-depleted and immunodepleted reconstituted vesicles were used in nuclear reassembly assays.

Nuclei reassembled in vitro from mock- or AKAP149-depleted vesicles were morphologically indistinguishable by phase–contract microscopy (Fig. 5 C). Both sets of nuclei contained nuclear membranes (Fig. 5 C, Phase), indicating that depletion of AKAP149 from NE-precursor vesicles did not hinder the assembly of these structures, and that integral membrane proteins required for NE assembly were successfully reconstituted. However, AKAP149 was not detected by immunofluorescence analysis of nuclei reassembled from AKAP149-depleted vesicles (Fig. 5 C). More importantly, PP1 was undetectable in these nuclei but was clearly localized at the NE of nuclei reassembled from mock-depleted control vesicles (Fig. 5 C). AKAP149 and PP1 were also detected in the NE of nuclei reformed from solubilized and reconstituted control vesicles not exposed to immunoglobulins (data not shown). We concluded that the absence of AKAP149 from nuclear membranes abolished the targeting of PP1 to the NE upon nuclear reassembly.

A

— PP1-BD   + PP1-BD   + PP1-BD (V155A)

Phase

AKAP149

PP1

B

Input   Depleted   Mock

anti-AKAP149

C

Mock-dep. ves.   AKAP149-dep. ves.

Phase

AKAP149

PP1

Figure 5. AKAP149 anchors nuclear PP1 at the NE. (A) Inhibition of PP1 recruitment to the NE by PP1-BD. Nuclei were reassembled in vitro as in the legend to Fig. 4; however, the interphase cytosol was preincubated with either no peptide, 10 μM of the PP1-binding competitor peptide PP1-BD, or 10 μM of control peptide PP1-BD(V155A). An ATP-generating system was added to promote nuclear reassembly at 30°C. After 2 h, the NE distribution of AKAP149 and PP1 was examined by phase–contrast and by immunofluorescence analysis using the anti-AKAP149 mAb and anti-PP1 polyclonal antibodies. (B) Immunodepletion of AKAP149 from mitotic membrane vesicles. Mitotic vesicles were solubilized with 0.5% NP-40/0.4 M KCl, and AKAP149 was immunodepleted by two successive rounds of immunoprecipitation. Mock depletions were performed with nonimmune mouse IgGs. AKAP149-depleted and mock-depleted vesicles were reconstituted by dialysis, washed, and aliquots were dissolved in SDS sample buffer. AKAP149 immunodepletion was monitored by immunoblotting each fraction. (C) PP1 is not recruited to AKAP149-depleted NEs. Nuclei were reassembled in interphase cytosol using membrane vesicles either mock-depleted (left) or immunodepleted of AKAP149 (right). Distribution of AKAP149 and PP1 was determined by immunofluorescence. Bars, 10 μm.

To definitely establish the role of NE-bound AKAP149 in recruiting PP1, we performed a rescue experiment allowing the reincorporation of AKAP149 into nuclear membranes. To this end, nuclei assembled with AKAP149-depleted vesicles were sedimented through a sucrose cushion and resuspended in fresh interphase cytosol. Exogenous PP1 was provided to the cytosol with recombinant PP1 (7×10^{-3} U/μl cytosol) to ensure that PP1 was not limiting. The cytosol contained either no exogenous vesicles, AKAP149-depleted vesicles (control), or mock-depleted vesicles harboring AKAP149. GTP was added to all extracts to promote vesicle fusion with existing nuclear membranes (Collas et al., 1996). The results are shown in Fig. 6. Without exogenous vesicles, or with AKAP149-depleted vesicles, no AKAP149 staining was detected and faint intranuclear PP1 labeling occurred. However, in cytosol harboring AKAP149-containing membranes, AKAP149 was clearly detected in the NE (no such staining occurred when GTP was omitted from the cytosol, indicating that AKAP149 was incorporated into the NE as a result of membrane fusion; data not shown). Furthermore, PP1 was exclusively detected at the NE where it colocalized with AKAP149. Importantly, NE targeting of PP1 was inhibited with 10 μM PP1-BD and thus was mediated by AKAP149. Collectively, these results indicate that incorporation of AKAP149 into AKAP149-deficient nuclear membranes restores targeting of PP1 to the NE, and dem-

onstrate that AKAP149 anchors PP1 to the NE upon nuclear reassembly in the extract.

Recruitment of PP1 to the NE Correlates with Lamin B Assembly

Reassembly of the nuclear lamina at the end of mitosis requires lamin dephosphorylation. Inhibition experiments, cofractionation of the lamin phosphatase activity and PP1, and affinity copurification of PP1 and lamin B phosphatase activity with microcystin-sepharose beads have previously pointed to PP1 as a mitotic lamin B phosphatase (Thompson et al., 1997). Additionally, we showed that lamin B assembly correlated with assembly of AKAP149 and recruitment of PP1 to the NE (see Fig. 4). These observations raise the hypothesis that targeting of PP1 to the NE is a prerequisite for the assembly of the nuclear lamina.

To address this issue, we examined by immunofluorescence the relationship between PP1 targeting to the NE and lamin B assembly into the NE in the course of three experiments performed earlier. The results are shown in Fig. 7. First, lamin B assembly was examined during nuclear assembly without any competitor peptide, with PP1-BD, or with the control PP1-BD(V155A) peptide. As shown earlier with the anti-PP1 polyclonal antibody (see Fig. 5 A), only PP1-BD abolished PP1 recruitment to the

PP1	+	+	+	+
AK-dep. ves.	-	+	-	-
Mock-dep. ves.	-	-	+	+
PP1-BD pep.	-	-	-	+
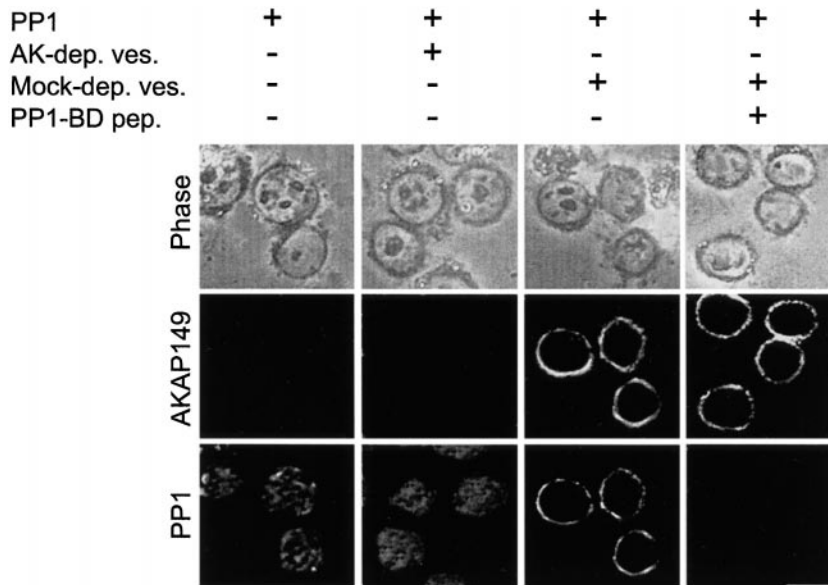
Figure 6. Restitution of AKAP149 in nuclear membranes restores recruitment of exogenous PP1 to the NE. Nuclei reconstituted with AKAP149-depleted vesicles as in Fig. 5 C were sedimented through sucrose and resuspended into fresh interphase cytosol containing combinations of recombinant PP1, AKAP149-depleted vesicles (control), mock-depleted vesicles (harboring AKAP149), and 10 μM of the PP1-binding competitor peptide PP1-BD. GTP was added to all reactions to promote fusion of vesicles with nuclear membranes. After 1.5 h, distribution of AKAP149 and PP1 was examined by immunofluorescence. Bar, 10 μm.

NE (Fig. 7 A). Remarkably, lamin B also remained undetected with PP1-BD, while its assembly occurred normally in control nuclei (Fig. 7 A). Second, nuclei reconstituted in vitro with AKAP149-depleted vesicles (see Fig. 5 C) did not harbor any detectable lamin B (nor PP1), whereas nuclei reassembled from mock-depleted vesicles clearly incorporated lamin B (Fig. 7 B). Third, in a rescue experi-

ment involving restitution of AKAP149 into AKAP149-depleted nuclear membranes (see Fig. 6), restoration of PP1 targeting to the NE also restored lamin B assembly (Fig. 7 C). In contrast, absence of NE-associated AKAP149, and thereby of PP1, or inhibition of PP1 recruitment to the NE by PP1-BD both abolished lamin B assembly (Fig. 7 C). It is noteworthy that the detection of nucleoli in nuclei
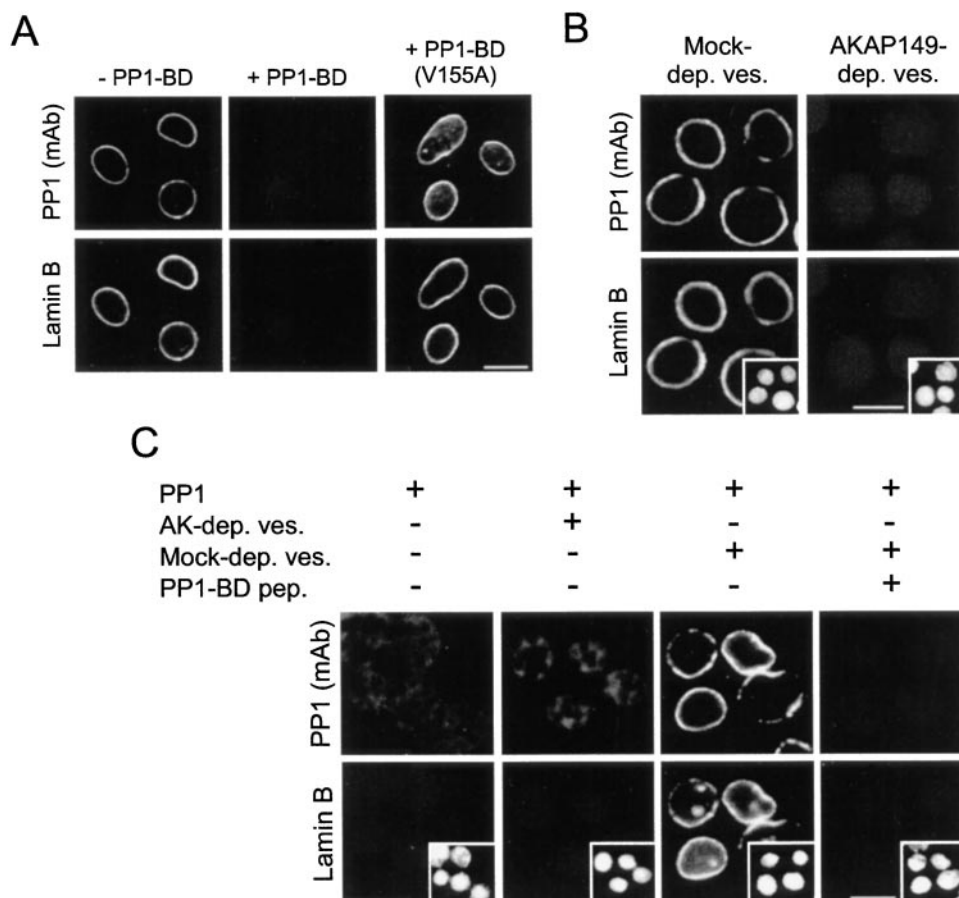
Figure 7. Targeting of PP1 to the NE by AKAP149 correlates with lamin B assembly. (A) PP1-BD prevents lamin B assembly. Nuclei were reassembled in the absence of competitor peptide, or with 10 μM PP1-BD or control PP1-BD(V155A), as in Fig. 5 A. Targeting of PP1 to the NE and assembly of lamin B were analyzed by immunofluorescence. (B) AKAP149-depleted nuclei do not assemble lamin B. Nuclei were reassembled from mock-depleted or AKAP149-depleted vesicles as in Fig. 5 B, and targeting of PP1 and lamin B to the NE was monitored by immunofluorescence as in A. (C) Restitution of AKAP149 in the NE restores lamin B assembly. Nuclei reformed from AKAP149-depleted vesicles as in B were exposed to fresh interphase cytosol containing combinations of PP1, AKAP149-depleted vesicles, mock-depleted vesicles, and PP1-BD as in the legend to Fig. 6. After 1.5 h, PP1 targeting to the NE and lamin B assembly were immunofluorescently examined. Insets, DNA staining with Hoechst 33342. Bars, 10 μm.

assembled in the presence of PP1-BD or from AKAP149-depleted vesicles (see Fig. 5) indicates that nuclear import did occur, ruling out the possibility that the absence of lamin B detection in these nuclei was a mere consequence of defective nuclear import. Rather, our results indicate that PP1 targeting to the NE by AKAP149 correlates with polymerization of B-type lamins into the lamina.

Discussion

We report a role of the PKA-binding protein AKAP149 as a targeting and anchoring protein for PP1 at the NE upon somatic nuclear reassembly in vitro. We also demonstrate a requirement of PP1 targeting to the NE for assembly of the nuclear lamina. Targeting of PP1 by AKAP149 supports a concept whereby AKAPs may constitute molecular platforms anchoring entire signaling units rather than PKA alone. This adds to the previous demonstration of subcellular targeting of PKA and/or PKC together with PP1 or PP2B by AKAP79 (Coghlan et al., 1995; Klauck et al., 1996), AKAP220 (Schillace and Scott, 1999), and yotiao/AKAP450/CG-NAP (Takahashi et al., 1999; Westphal et al., 1999; Witczak et al., 1999).

AKAP149 Is an Integral Membrane Protein of the NE

We identified AKAP149 as an integral protein of the ER and NE membranes, with a NH$_2$-terminal transmembrane domain and the bulk of the protein facing the cytoplasm. Recruitment of chromatin-bound PP1 to the NE by AKAP149 argues that AKAP149 is also anchored in the inner nuclear membrane. This is consistent our observation that A/C-type lamins coimmunoprecipitate with AKAP149 from solubilized NE preparations (Steen, R.L., and P. Collas, unpublished data). To our knowledge, this makes AKAP149 the first example of a cellular integral protein localized in both nuclear membranes rather than being restricted to either membrane domain (see Worman and Courvalin, 2000). Sequence information and topology studies suggest that the cytoplasmic or nucleoplasmic domain of AKAP149 approximates 147 kD, which by far exceeds the size limit allowing the diffusion of membrane proteins through the nuclear pore complex (Soullam and Worman, 1995). Thus, it is unlikely that AKAP149 diffuses from the ER to the inner nuclear membrane during interphase. Rather, a portion of ER-bound AKAP149 may become restricted to the inner nuclear membrane upon targeting to chromatin when nuclear membranes reform at the end of mitosis, while the rest of AKAP149 remains in the ER/outer nuclear membrane network.

Anchoring of PP1 at the NE by a PKA-binding Protein

Two major nuclear regulatory subunits of PP1 have previously been identified, nuclear inhibitory subunit of PP1 (NIPP1) (Jagiello et al., 1995) and PP1 nuclear targeting subunit (PNUTS) (Allen et al., 1998), which have been proposed to target PP1 to RNA (Jagiello et al., 1997). AKAP149 also binds PP1 in vitro and in vivo and harbors a KGVLF domain in its NH$_2$-terminal domain, which falls within known PP1-binding consensus motifs (Zhao and Lee, 1997). Based on these criteria, we propose that AKAP149 constitutes a novel PP1 targeting protein. How-

ever, whether AKAP149 also acts as a regulatory subunit of PP1 remains undetermined. Affinity purification and coimmunoprecipitation experiments have identified NIPP1 in the AKAP149–PP1 complex in vivo (Steen, R.L., and P. Collas, unpublished data). This suggests that AKAP149 functions as a targeting protein for the PP1–NPP1 holoenzyme, with no regulatory activity. Alternatively, in addition to its targeting role, AKAP149 may regulate PP1 activity together with NIPP1 (synergistically or antagonistically) at the level of the NE. These hypotheses remain to be tested.

Remarkably, the KGVLF domain of AKAP149 is flanked by four serines, raising the possibility that their phosphorylation by a Ser/Thr kinase regulates AKAP149–PP1 interaction. To support this view, phosphorylation of serines flanking the RVXF motif of NIPP1 by PKA and casein kinase 2 affects the association of PP1 with NIPP1 (Beullens et al., 1993). Furthermore, PKA-mediated phosphorylation of the PP1 regulatory subunit G$_M$, which targets PP1 to glycogen in skeletal muscle, also abolishes PP1 binding (Egloff et al., 1997). Thus, phosphorylation of PP1 regulatory subunits may modulate PP1 activity by altering its binding to the regulatory subunit.

We have shown that NE-associated AKAP149 not only anchors PP1 but also binds PKA in vitro. We cannot at present ascertain whether AKAP149 anchors PKA and PP1 in distinct complexes or in a single ternary complex, but in either situation, implications of a PKA-binding protein anchoring a phosphatase at the NE are several. First, AKAP149 may conceivably position PKA and PP1 in close proximity where they can reversibly modulate the phosphorylation of nuclear substrates such as NPP1 (Beullens et al., 1993), DNA-binding cAMP response elements (Riabowol et al., 1988), B-type lamins (Peter et al., 1990), and inner nuclear membrane proteins LBR and lamina-associated polypeptides, which all harbor PKA phosphorylation sites. Phosphorylation/dephosphorylation of B-type lamins in interphase (Kill and Hutchison, 1995) may be implicated in the regulation of NE–chromatin interactions, which in turn may regulate intranuclear processes such as replication of heterochromatin or redistribution of B-type lamins from the NE to foci of DNA replication (Ellis et al., 1997). Second, as no PKA regulatory subunit has been detected in the nucleus (Eide et al., 1998), AKAP149 may also target the PKA holoenzyme at the NE (presumably on the cytoplasmic face of the outer nuclear membrane) to enhance the efficiency of translocation of activated PKA catalytic subunits to the nucleus in response to cAMP signaling (Riabowol et al., 1988). Finally, PKA may constitute a regulator of AKAP149–PP1 binding by altering AKAP149 phosphorylation. Indeed, association of AKAP149 with PP1 is cell cycle regulated, as AKAP149 remains associated with ER membranes at mitosis (Fig. 1) while PP1 is bound to chromatin (Fernandez et al., 1992).

AKAP149 and NE-associated PP1 Are Implicated in Nuclear Lamina Assembly

Our observations suggest that NE-associated PP1 promotes lamina assembly at the end of mitosis. PP1 has been shown to be the major lamin B phosphatase at mitosis exit (Thompson et al., 1997), such that NE-associated PP1 may dephosphorylate B-type lamins before their assembly into

the lamina. Consistent with this view is our observation that targeting of PP1 to the NE by AKAP149 upon nuclear reformation correlates with lamin B assembly. Phosphorylation of avian lamin B2 by PKC has been shown to impair nuclear import of the lamin (Hennekes et al., 1993). If this holds true in mammals, dephosphorylation of PKC sites by an as yet unidentified cytoplasmic phosphatase should precede nuclear import of B-type lamins. PP1 anchored at the nuclear membrane may subsequently dephosphorylate additional (non-PKC) residues after nuclear import of the lamins, albeit before their polymerization into a lamina.

In addition to providing structural support to the nucleus, lamins have been proposed to assume a variety of nuclear functions (Ellis et al., 1997; Jagatheesan et al., 1999). Most significantly, two hereditary disorders, Emery-Dreifuss muscular dystrophy and a lipodystrophy (Dunningan's disease), have been shown to correlate with mutations in the *Lmna* (lamin A/C) gene (Bonne et al., 1999; Cao and Hegele, 2000), and *Lmna*$^{-/-}$ mice develop a pathology analogous to Emery-Dreifuss muscular dystrophy (Sullivan et al., 1999). Interestingly, the nuclei of *Lmna*$^{-/-}$ mice are deformed and exclude B-type lamins specifically in the deformed areas (Sullivan et al., 1999). This suggests that interactions between B-type lamins and the inner nuclear membrane or chromatin, or the polymerization state of lamin B, are altered in these regions. It will be relevant to examine the mechanism underlying B-type lamin distribution in affected nuclei and whether AKAP149 is implicated in the mislocalization of these lamins.

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